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14. ABSTRACT

We reported last year that ER K303R mutation makes ER serine 305 an efficient target for both Protein Kinase A (PKA) and p21 activating kinase (PAK1), thus confers breast cancer cells expressing this mutant hypersensitivity.we report that the K303R mutant is also an efficient target for AKT activity. We have localized that Ser305 is an Akt site. In K303R ERa, this site is about 3 times more phosphorylated by Akt than ER Ser167, a known AKT phosphorylation site, and about 30 times more phosphorylated than Ser305 in wild type ERa. We utilized the mammalian two-hybrid system to test the effect of Akt activity on the ligand-dependent activity (AF2) of wild type and K303R ER. Our data demonstrated that constitutively activated Akt2 could specially enhance the estrogen –hypersensitivity of K303R. Also, we found that AKT activity reverses MTA2 repression of WT-ER activity. Our results demonstrate that the K303R ERa mutation generates a novel Akt site which renders the receptor hypersensitive.

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INTROUCTION

It is known that estrogens play a key role in regulating the proliferation of normal breast epithelium, as well as breast cancer, through binding with their cognate receptors (ERs), which belong to a superfamily of nuclear steroid receptors and function as transcriptional factors to mediate the mitogenic effects of estrogens. It has been reported (1,2) that less than 10% normal breast epithelium express ERα, but that about 60% of UDH and 60-70% of breast tumors express ERα, so it is felt that the expression and/or activation of ER influences both breast carcinogenesis and progression. There are two current models for ER activation. One is through its cognate ligand binding; the other involves alternative pathways such as phosphorylation, acetylation, or mutation. Recently our group (3) has discovered that a mutated estrogen receptor called K303R, is correlated with with both breast cancerignesis and development, and also this mutant is a hypoacetylated ER form, so that we proposed to study the effect of an HDAC-associated protein, MTA1-L1 mediated deacetylation of wild-type ER function, as well as to study the effects of expressing the K303R mutant on breast cancer cell metastatic behavior in the following Specific **Aims**:

Aim 1. To elucidate the relationship between MTA-L1 expression, and ERα protein acetylation as well as ERα activity: We have already shown an *in vitro* interaction between wild-type ERα and MTA-L1 using GST-pull down experiments. To detect the potential significance of this interaction, we will examine for *in vivo* interactions as well as the effects of ERα agonists and antagonists on this interaction using further GST-pull down and co-immunoprecipitation assays. The effects of MTA-L1 expression on ERα transcriptional activity will also be determined using transient transactivation assays and estrogen-responsive reporter assays. The effects of MTA-L1 expression on ERα acetylation status will be determined using *in vitro* deacetylation assays.

Aim 2. To study the metastatic process influenced or driven by the K303R ERα mutation, and to develop a somatic knock-out model of the ERα gene in the T47D cell line to study the *in vivo* effects of the K303R mutation. A somatic knock-out of the human ERa gene will be performed using standard approaches targeted to the amino region or the DNA binding domain. The functional significance of the ERα mutation will first be determined using *in vitro* motility and invasion assays of the knock-out cell line transfected with an expression vector for the wild-type, the K303R mutant, or co-expressed MTA-L1. The *in vivo* metastatic potential and behavior of the transfected lines will then be examined using both ectopic growth and tail vein injection into athymic nude mice. Metastatic lesions, if present, will be examined for WT ER, K303R ER, nd/or MTA-L1 expression using microdissection, genomic sequence, and SNP analyses. The sensitivity of the metastatic lesions to estrogens antiestrogens will be examined to determine their hormonal response to treatment with these agents.

BODY

Specific Aim 1

we have successfully finished and have dramatically extended the studies proposed in **Specific Aim 1** (see 2004 Annual Report)

Specific Aim 2

we also successfully finished some *in vitro* studies proposed in **Specific Aim 2** last year (see 2004 Annual Report) and swithched **Specific Aim 2 to the following studies(See 2004 Annual Report : CONCLUSIONS) .**

The Breast Cancer Specific K303R Estrogen Receptor a Mutation Generates an Integral Site for AKT Signaling

Since it has been shown recently that PI3 Kinase and its downstream target, Akt, are involved in breast tumor progression and therapeutic response, possibly through their effects on cell survival. We have therefore examined the effect of Akt on wild-type and K303R ERa activity. Using an immune complex kinase assay with an antibody that recognizes Akt, we found that the K303R mutant exhibited more phosphorylation(Fig 1). Using different site mutations as the substrate, we localize that Ser305 is an Akt site since mutation of 305S to D in the K303R ERa construct, prevented *in vitro* phosphorylation by Akt1(Fig 2). The Ser 305 in K303R ERa is about 3 times more phosphorylated by Akt than the known Ser167, and about 30 times more phosphorylated than Ser 305 in wild type ERa (Fig 3), suggesting that this K303R mutation switchs the major acting site of Akt from the ligand-independent activation function (AF1) to the ligand-dependent function (AF2). To address this hypothesis, we utilized the mammalian two-hybrid system to test the effect of Akt activity on the ligand-dependent activity (AF2) of wild type and K303R ER. Our data demonstrated that constitutively activated Akt2 could specially enhance the estrogen –hypersensitivity of K303R (Fig 4) Also we demonstrated that co-transfection of dominant-active AKT2 reverse MTA2 inhibited ERα activity.Our results demonstrte that the K303R ERa mutation generates a novel Akt site which renders the receptor hypersensitive

KEY RESEARCH ACCOMPLISHMENTS

1. Demonstrated that Ser 305 is a novel AKT phosphorylation site within ERα
2. Demonstrated that inhibition K303R mutation generates an efficient integral site for crosstalking between estrogen and PI3K/AKT signals
3. Demonstrated that both K303R mutation switches the major acting site of Akt from the ligand-independent activation function (AF1) to the ligand-dependent function (AF2).
4. Demonstrated that AKT activity could release MTA2 repression of ER activity.

REPORTABLE OUTCOMES

1. (1) meeting abstract, 1 paper included in the Appendix.
2. Figues and relevant figure legends, were included in Appendix

CONCLUSIONS

We have successfully completed the proposed **Specific Aim1**, and a part that proposed in **Specific Aim2** Results on this project have generated (2) manuscripts (one published in *Cancer Res* , one was resubmitted to *Mole Endo* after revised) and (1) meeting abstract which was selected as Poster Discussion in 2004 *San Antonio Breast Cancer Symposium*. We want to state that **we have dramatically extended the studies proposed in Specific Aim 1,** and generated more additional exciting results with potential translational significance. The proposed studies will continue to be studied in the laboratory of the mentor, Suzanne A.W. Fuqua. Undoubtedly, the funds for this fellowship have been well spent with much success. It is also conceivable that this line of study will lead to direct clinical benefit.

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APPENDICES

Reprints 1: Yukun Cui, Mao Zhang, and Suzanne AW Fuwua. The Breast Cancer Specific K303R Estrogen Receptor a Mutation Generates an Integral Site for AKT Signaling. Breast Cancer Research and Treatment. 2004 88(Suppl 1): S27
Reprints 2: Yukun Cui, Mao Zhang, Richard Pestell, Edward M. Curran, Wade V. Welshons and Suzanne A. W. Fuqua. 2004 Phosphorylation of estrogen receptor alpha blocks its acetylation and regulates estrogen sensitivity. Cancer Research 64(24):9199-208

Construction of Plasmids- S305D, and S294A ERα constructs were generated using QuickChange Site-Directed Mutagenesis (Strategene, La Jolla, CA) to generate the indicated point mutations in pcDNA3.1-HA-WT ERα. The primer sequences for construction of the point mutations are as follows, with the mutated nucleotides underlined:
GCTCTAAGAA(G)GAACGACCTGGCCTTGTCCTGACG; S305D,
GCTCTAAGAA(G)GAACGCCCTGGCCTTGTC CTGACG; S294A, GCCAACCT
TTGGCCGACCCCGCTCATGATCAAACG;; K303R/S305D,
GATCAAACGCTCTAAGAGGAACGCCCTGGCCTTG . The entire nucleotide sequence of the construct was then verified by standard DNA sequencing procedures. The bacterial expression vectors for GST fusion proteins of the ERα hinge region (residues 253-310) were constructed by cloning PCR-amplified fragments derived from their respective mammalian vectors. To generate Gal4-ER constructs, ER fragments (251-595) were was amplified from relevant pcDNA3.1 ERαs and inserted into pBinder vector through Asp718 and Bam H1 sites..

Figure Legends

Fig. 1. K303R mutation generates an efficient AKT phosphorylation site. Equal amount of GST-ERα Hinge proteins with or with out K303R mutation were subjected to phosphorylation with recombinant PKA, AKT1 (PKB) or PKC. The resultant proteins were resolved onto SDS-PAGE and transferred onto nitrocellular membrane and immunoblotted with anti-phospho serine antibody (IF8) and visualized with ECL reagent.

Fig.2. Identification Serine 305 as a novel AKT phosphorylation site. Equal amount of GST-ERα Hinge proteins harboring K303R or K303R/S305D mutations were subjected to phosphorylation with recombinant AKT1 in the presence of p32-gamma ATP. (PKB). The resultant proteins were resolved onto SDS-PAGE and visualized with autoradiography.

Fig.3. K303R mutant makes renders ERα Serine 305 the most important phosphorylation site for AKT activity. GST GST-ERα Hinge proteins with or with out K303R mutation were subjected to

phosphorylation with increasing amounts of recombinant AKT1 in the presence of p32-gamma ATP. (PKB). The resultant proteins were resolved onto SDS-PAGE and the incorporated p32 were visualized with autoradiography. GST-AF1 proteins phosphorylated by fixed amount of AKT1 was used to assay the relative phosphorylation efficiency. Upper panel is autoradiography, lower panel is the commassie blue staining of the same gel to assay the substrates used in this experiment.

Fig.4. K303R mutation confers ERα ligand hypersensitivity in the presence of dominant active AKT2. U2OS cells were cotransfected with 1 μg pG5-Luciferase reporter plasmid (Promega, Wisconsin, MI) , plus 10 ng of the respective Gal4-ERα AF2 expression plasmids, 25 ng dominant active AKT2, and 100 ng of CMV-β-galactosidase expression plasmid as an internal control. Twenty-four hours after transfection, wells were then treated with increasing amounts of E_2 (10^{-11} to 10^{-9} M).Cells were harvested after 18-24 hours of treatment with hormone, and ER luciferase activity was normalized by dividing by the β-galactosidase acitivity to give relative luciferase units. Experiments were performed in triplicate; the data are presented as the average +/- SEM and are representative of at least three independent experiments.

Fig.5. AKT activity release MTA2 repressed ERα activity. MCF-7 cells were grown in phenol red free medium plus 5% stripped serum were cotransfected with 1 μg ERE-Luciferase reporter, plus 800 ng of the MTA2 expression vector or empty vector with or without cotransfection of 200 ng dominant active AKT2, and 100 ng of CMV-β-galactosidase expression plasmid as an internal control. Twenty-four hours after transfection, wells were then treated with 1nM of E_2 .Cells were harvested after 18-24 hours of treatment with hormone, and ER luciferase activity was normalized by dividing by the β-galactosidase acitivity to give relative luciferase units. Experiments were performed in triplicate; the data are presented as the average +/- SEM and are representative of at least three independent experiments.

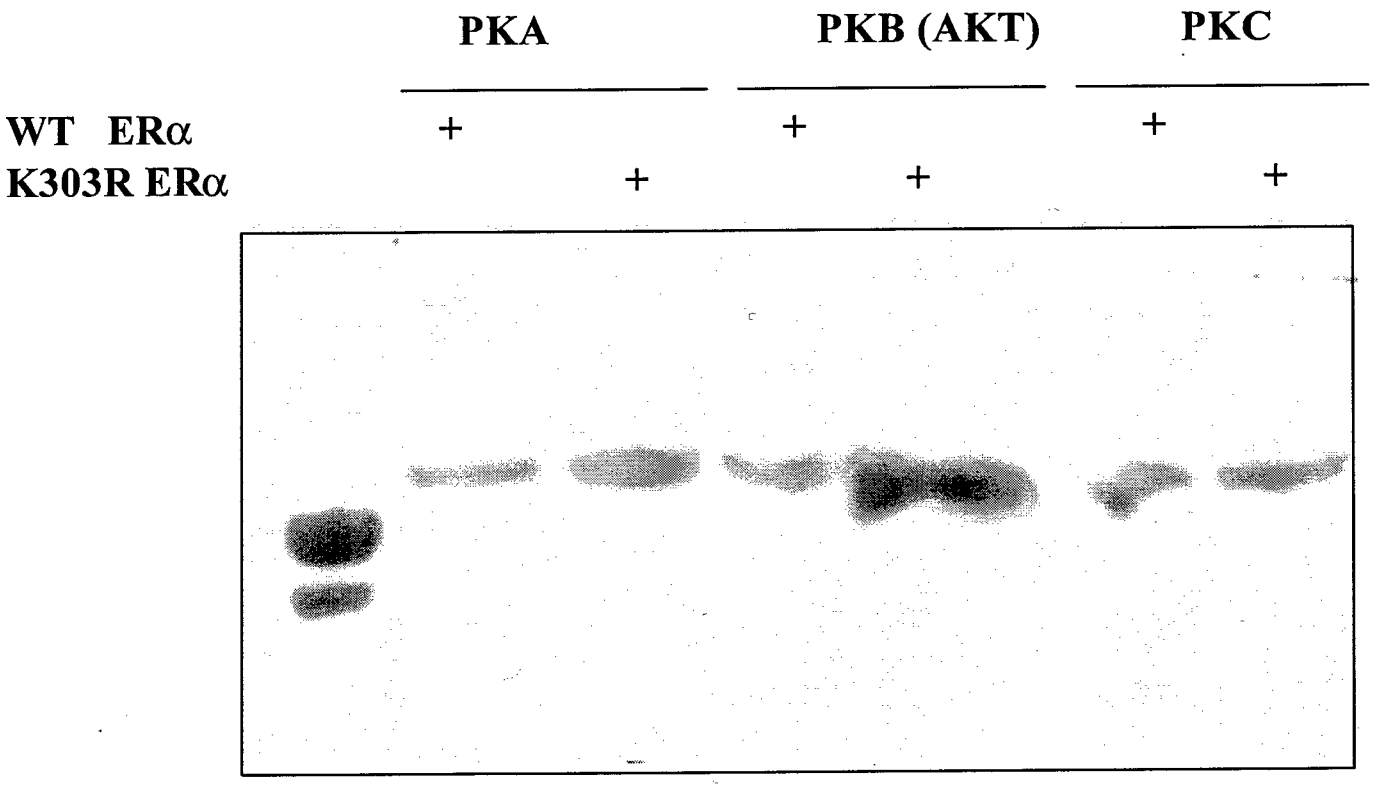
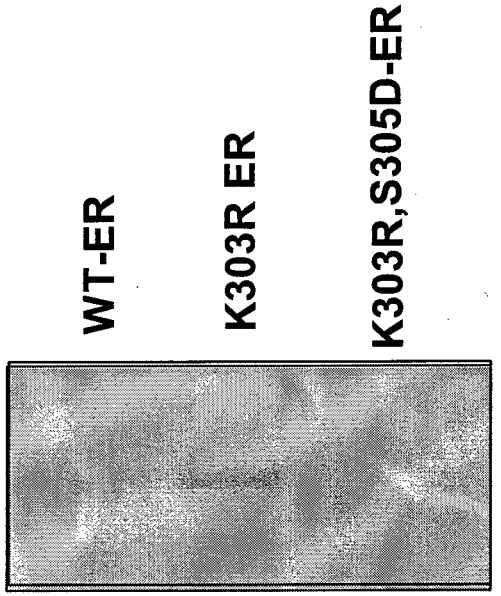


Fig.1



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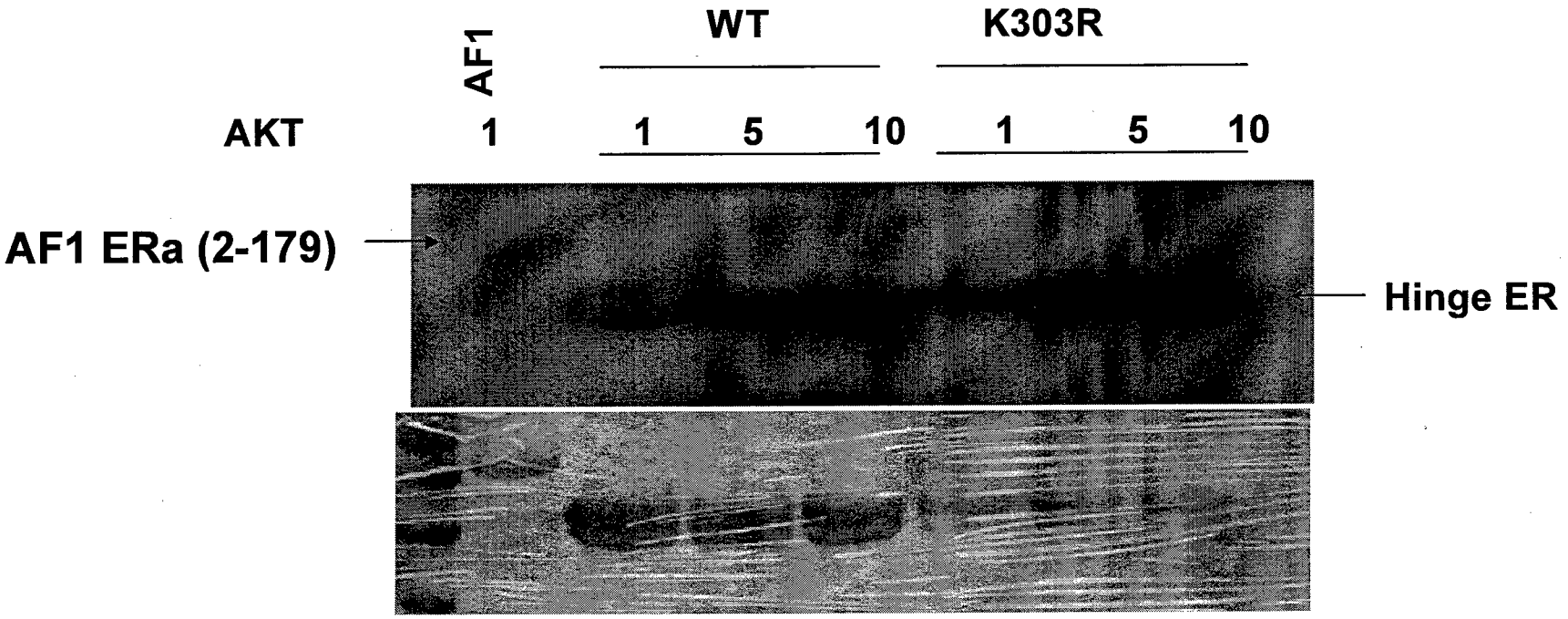

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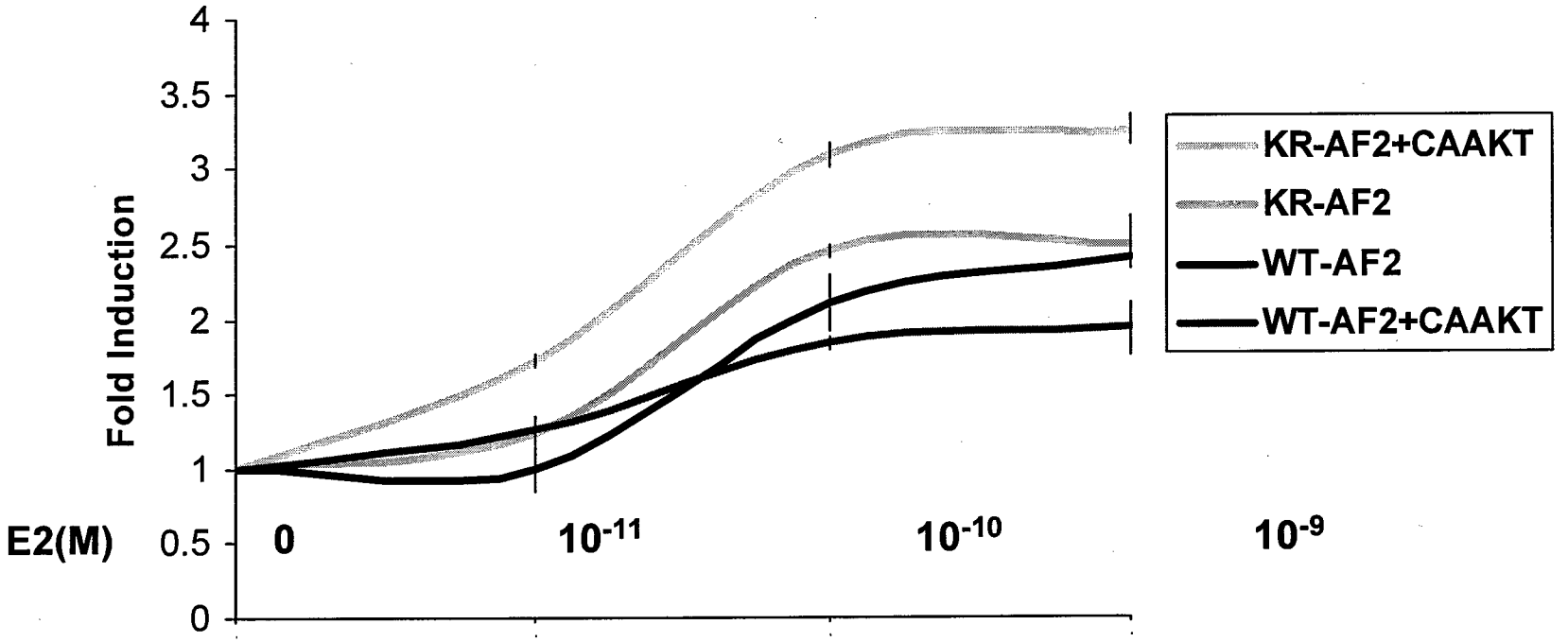
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KR-AF2
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WT-AF2+CAAKT

Fig 4

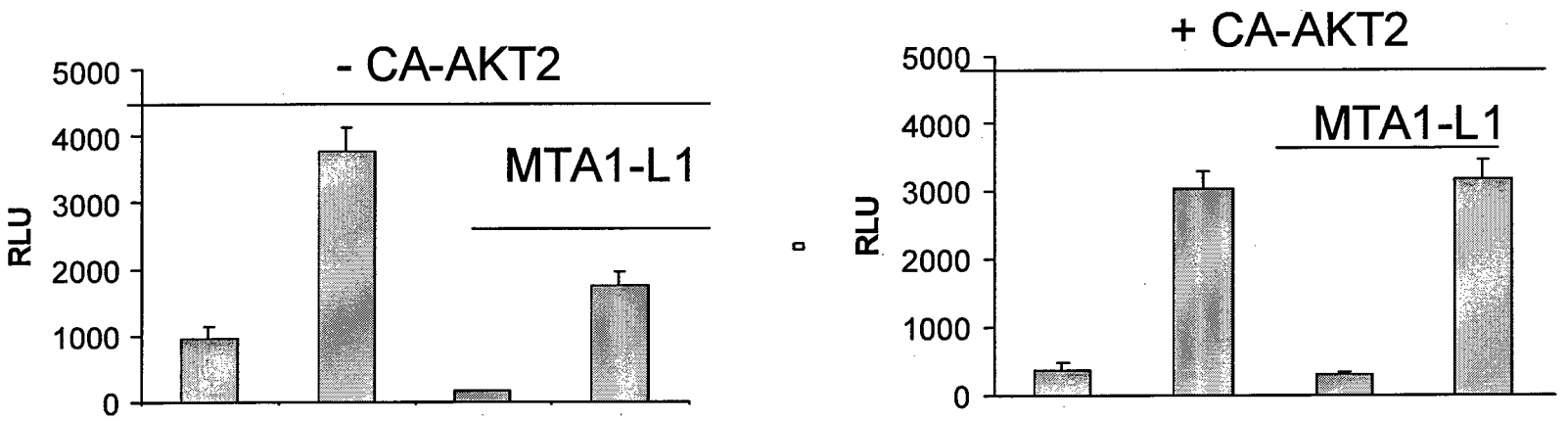
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Fig 5